

Morpho-Electric Properties and Diversity of Oxytocin Neurons in Paraventricular Nucleus of Hypothalamus in Female and Male Mice

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Oxytocin (OXT) neurons in paraventricular nucleus of hypothalamus (PVN) are involved in modulating multiple functions, including social, maternal, feeding, and emotional related behaviors. PVN OXT neurons are canonically classified into magnocellular (Magno) and parvocellular (Parvo) subtypes. However, morpho-electric properties and the diversity of PVN OXT neurons are not well investigated. In this study, we profiled the morpho-electric properties of PVN OXT neurons by combining transgenic mice, electrophysiological recording, morphologic reconstruction, and unsupervised clustering analyses. Total 224 PVN OXT neurons from 23 mice were recorded and used for analyses in this study, and 29 morpho-electric parameters were measured. Magno and Parvo OXT neurons have prominent differences in their morpho-electric features, and PVN OXT neurons in male and female mice share similar neuronal properties. Some morpho-electric features of PVN OXT neurons, especially Magno neurons, exhibit significant diverse changes along the rostral–caudal axis. Furthermore, we find that PVN OXT neurons are classified into at least six subtypes based on their morpho-electric properties via unsupervised clustering. Only one Magno-Parvo mixed subtype in posterior PVN subregion, but not the other five subtypes, showed significant neuronal activity change in different feeding conditions. Our study supports the diversity of PVN OXT neurons and subtle neuron classification will promote excavating the functions of oxytocinergic system.

Key words: classification; feeding; morpho-electric properties; oxytocin; PVN

Significance Statement

Oxytocin (OXT) is well known for its function in labor induction, but it also plays multiple roles in social, feeding, and emotional behaviors via modulating different brain regions. Paraventricular nucleus of hypothalamus (PVN) OXT neurons are traditionally classified into magnocellular and parvocellular. However, functional and single-cell transcriptomic studies indicate that OXT neurons should be further classified. Here, we thoroughly investigated the morpho-electric properties and spatial distribution of PVN OXT neurons, and find that OXT neurons have at least six subtypes based on their morpho-electric features. Among these six subtypes, only one magnocellular-parvocellular mixed subtype, which are distributed in the posterior PVN subregion, change their activities with different feeding states. Our study uncovers the diversity of PVN OXT neurons and suggests the necessary of subtle neuronal classification.

Introduction

Neuron is the basic unit of CNS. Mammalian brain comprises millions of neurons, which are categorized into diverse subtypes according to their morphologic, electrophysiological, and molecular properties. Neuronal diversity determines the functional complexity of the CNS. With the development of transgenic and molecular tools and techniques, more types of neurons in cortex and subcortical regions are gradually uncovered (Saunders et al., 2018; Gouwens et al., 2020). These studies have advanced our understanding about the refined brain structures and the elegant neural networks for controlling our motion, cognition, and emotion. Paraventricular nucleus of hypothalamus (PVN), which is a key center for neuroendocrine and autonomic regulation,

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consists of several neuropeptide releasing neurons, including oxytocin (OXT), vasopressin, corticotropin releasing hormone, and so on. PVN neurons have a broad projection in the brain, and are involved in a lot of innate and learned behaviors (Knobloch et al., 2012; Jirikowski, 2019). To better understand the functions and mechanisms, the diversity of PVN neurons should be further investigated (Althammer and Grinevich, 2017; Romanov et al., 2017; Xu et al., 2020).

Based on the projections and electrophysiological properties, PVN neurons are canonically divided into two subtypes: magnocellular (Magno) and parvocellular (Parvo) (Hoffman et al., 1991; Tasker and Dudek, 1991; Luther and Tasker, 2000; Stern, 2001). Magno, but not Parvo, neurons project to the posterior pituitary and release neurohormone into the blood. In electrophysiological properties, Magno neurons exhibit a pronounced transient outward rectification and the A-type potassium current, whereas Parvo neurons express T-type calcium current (Tasker and Dudek, 1991; Luther and Tasker, 2000). OXT neuron is one main class of PVN neurons. In addition to the well-known projection to posterior pituitary and releasing OXT into blood circulation, PVN OXT neurons also project to many brain regions to modulate prosocial, feeding, maternal, and emotion-related behaviors (Dolen et al., 2013; Marlin et al., 2015; Hung et al., 2017; Carcea et al., 2021). Several recent studies assessed the analgesic properties and behavioral role of Parvo OXT neurons in rats and mice (Eliava et al., 2016; Hasan et al., 2019; Lewis et al., 2020; Tang et al., 2020). Most studies about PVN OXT neurons follow the canonical principle to simply classify OXT neurons into Magno and Parvo subtypes (Xiao et al., 2017; Lewis et al., 2020). However, recent studies about both neural circuit function and single-cell RNA sequencing suggested that PVN OXT neurons have more than two subtypes (Althammer and Grinevich, 2017; Romanov et al., 2017).

Substantial studies suggested the anorexigenic effect of OXT in brain (Sabatier et al., 2013; Romano et al., 2020). OXT infusion into brain decreased food intake and increased energy expenditure to reduce body weight (Arletti et al., 1989; Deblon et al., 2011; Blevins et al., 2015). Food intake will increase the activity of PVN OXT neurons via activation of vagal afferents, which in turn promotes OXT release (Yamashita et al., 2013). However, the role of PVN OXT neurons in regulating feeding behavior remains controversial. Direct ablating PVN OXT neurons in adult mice had no effect on body weight, food intake, or energy expenditure on a regular diet (Wu et al., 2012; Xi et al., 2017). Activation of PVN OXT neurons alone in food-deprived mice had no significant effect on food intake (Atasoy et al., 2012; Sutton et al., 2014), but targeted ipsilateral activation of PVN OXT neurons suppresses the feeding behavior induced by activating agouti-related peptide neurons in arcuate nucleus (Atasoy et al., 2012). These studies disrupted or modulated the whole PVN OXTergic system. Some studies suggested that projection of PVN OXT neurons to nucleus of the solitary tract (NTS) is responsible for leptin-induced body weight reduction (Perello and Raingo, 2013), and knockdown of NTS OXT receptors increased food intake (Ong et al., 2017). These results suggest that a specific subtype of PVN OXT neurons may be responsible for food intake regulation.

To dissect the diversity of PVN OXT neurons, we combined physiology, morphology, and clustering analyses. We first classified PVN OXT neurons into Magno and Parvo neurons, following the canonical principle, and thoroughly compared the differences between Magno and Parvo OXT neurons and along the rostral–caudal axis. With unsupervised clustering analyses,

we find that PVN OXT neurons are classified into at least six subtypes based on their properties, and one subtype significantly changed their activities with different feeding conditions.

Materials and Methods

Mouse strains and genotyping. Animals were handled following the protocols approved by the Fudan University Animal Care and Use Committee. Unless otherwise stated, mice were housed on a 12:12 light–dark cycle (8:00 A.M. light ON and 8:00 P.M. light OFF) with *ad libitum* access to food and water. Nonbreeding male and female mice (postnatal 85–97 d) were used in this study. B6.129S-Oxt^{tm1.1(cre)Doss}/J mice (Oxt-Cre, #024234, The Jackson Laboratory) were used to label OXT neurons. The floxed eYFP (Ai3) reporter strain was crossed with Oxt-Cre mice to visualize PVN OXT neurons. Mouse genotyping was conducted following standard procedures on The Jackson Laboratory websites.

Behavioral treatment. Before electrophysiology recording, mice were randomly pretreated in three different feeding conditions (Fig. 1): normal feeding (Normal), fasting (Fasting), refeeding after fasting (Refeeding). Mice were single housed in a new cage for 24 h with *ad libitum* access to food and water before feeding treatment. Then, mice were transferred into a new cage for different feeding treatments. For the Normal group, mice were housed with food and water for 25 h. For the Fasting group, mice were fasted for 25 h. For the Refeeding group, mice were provided with enough food and refed for 1 h after 24 h fasting, and mice were confirmed to consume the food during the refeeding time. Mice were killed for electrophysiological recording around the same time every day.

Acute slice preparation and electrophysiological recording. Acute brain sections were prepared from male and female Oxt-Cre: Ai3 mice (postnatal 85–97 d) as previously described (Xiao et al., 2017, 2018). Briefly, mice were deeply anesthetized by isoflurane, and then transcardially perfused with ice-cold, oxygenated (95% O₂/5% CO₂) ACSF (in mM) as follows: 127 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 2 CaCl₂, 1 MgCl₂, and 25 glucose (osmolality ~310 mOsm/L). Brain was quickly removed and placed into a slicing chamber containing ice-cold ACSF, bubbled with 95% O₂/5% CO₂. Coronal slices (~250 μm) were cut on a Vibratome 1000PLUS, and slices including PVN region were collected and incubated in oxygenated ACSF for ~20 min at 34°C before recording.

For electrophysiological recording, a slice was transferred to a recording chamber with constant perfusion with oxygenated ACSF at a flow rate of 1.5–2 ml/min, with temperature maintained at ~30°C during recording by a feedback inline heater (TC-324C; Warner Instruments). PVN OXT neurons were visualized in slices using an IR-DIC microscopy, and identified based on the eYFP signal. Current-clamp recordings were established with glass pipettes (3–5 MΩ) containing the following (in mM): 135 K-gluconate, 4 KCl, 10 HEPES, 10 Na-phosphocreatine, 4 MgATP, 0.4 Na₂GTP, and 1 EGTA, pH 7.2–7.3 (osmolality ~295 mOsm/L); 0.5% (5 mg/ml) biocytin was added into the internal solution for morphologic detection after recording. PVN OXT neurons with 30–40 μm depth away from slice surface were recorded, and every neuron was recorded ~10 min after break-in to ensure the biocytin filling. To minimize the severing of dendritic processes during the recording, glass pipettes approached the OXT neurons above the soma, but not laterally. Recordings were made using the 700B amplifier; data were digitized at 10 kHz and filtered at 4 kHz and collected using pCLAMP software (Molecular Devices).

Tissue processing, immunohistochemistry, and imaging. Mice were deeply anesthetized with isoflurane and perfused transcardially with 4% PFA in 0.1 M PBS. Brains were postfixed for 24 h in 4% PFA at 4°C, and then sectioned at 50 μm on a vibratome (VT1200, Leica Microsystems). Tissues including PVN region were chosen and pretreated in 0.2% Triton X-100 for 1 h at room temperature (RT), then blocked with 0.05% Triton X-100, 10% BSA in PBS for 1 h at RT and rinsed in PBS. Tissues were transferred into primary antibody solution (rabbit anti-OXT, 1:1000, T-4084, Peninsula Laboratories) in PBS with 0.2% Triton X-100 and incubated for 24 h at 4°C. Tissues were rinsed in PBS for 3 times, and incubated with secondary antibody solution (goat anti-rabbit

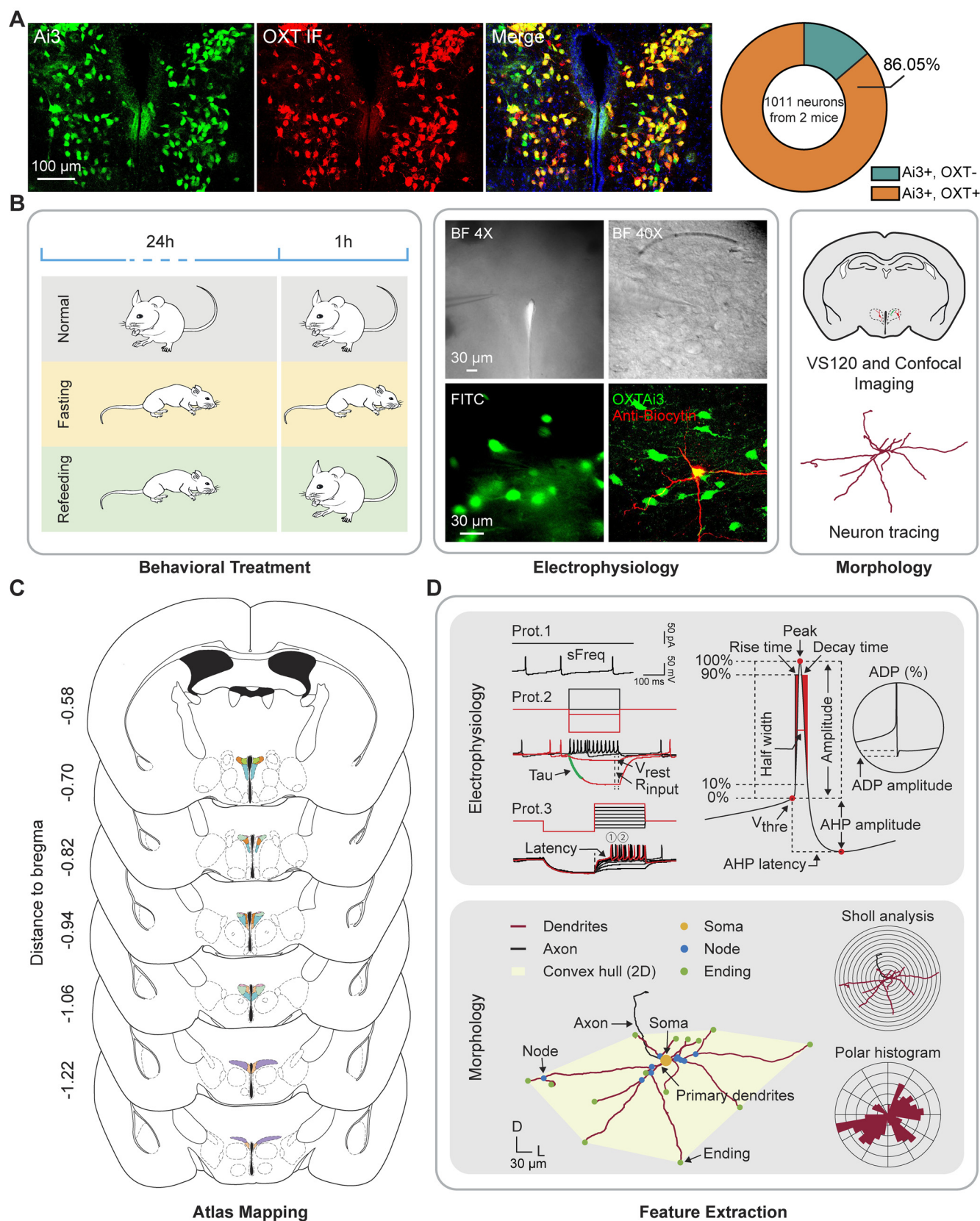


Figure 1. Experimental and data analyses procedures. **(A)** Left, Example images showing fluorescence expression in Oxt-Cre; Ai3 mouse (green), OXT immunostaining (red), and overlap between Ai3 signal and OXT IF. Right, Percentage of Ai3-positive neurons colocalized with OXT IF positive neurons. $n = 1011$ neurons from 2 mice. **(B)**, Experimental procedure. Mice were treated in three different feeding conditions (left), then PVN OXT neurons were recorded and labeled with biocytin (middle), and neuronal morphology and spatial location were imaged and reconstructed (right). **(C)**, The Paxinos brain reference atlas used to map the recorded PVN OXT neurons. **(D)**, Electrophysiological (top) and morphologic (bottom) properties extracted and analyzed in this study. ADP (%) indicates the probability of ADP occurrence. ①, ② indicate IS1 and IS2, respectively; D, dorsal; L, lateral to third ventricle.

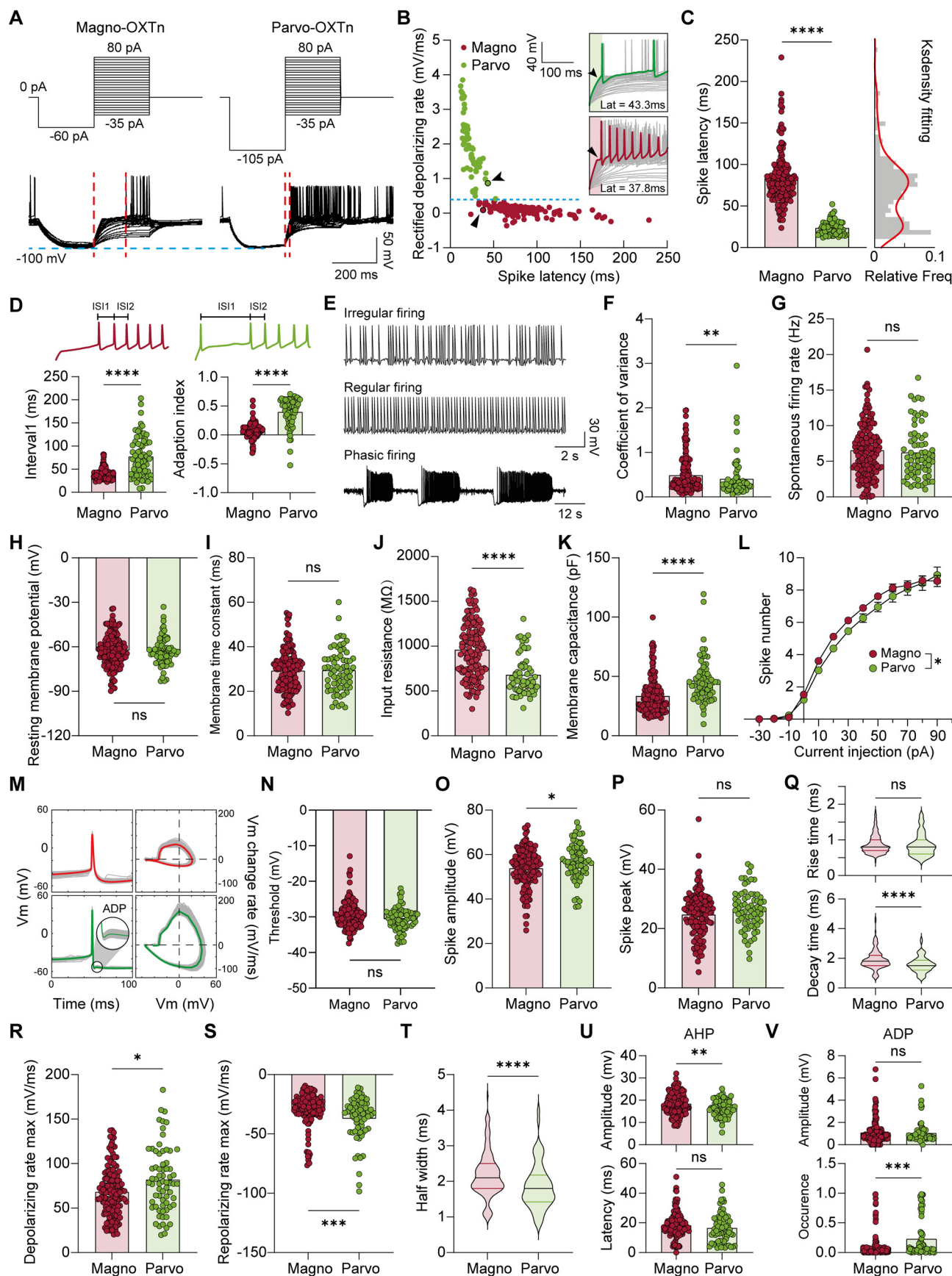


Figure 2. Physiologic properties of PVN OXT Magno and Parvo neurons. **A**, Representative traces from Magno (left) and Parvo (right) PVN OXT neurons. Top, Current injection protocols. Bottom, Neuronal responses. Blue dashed line indicates -100 mV membrane potential level. The duration between red dashed lines is the spike latency. **B**, Distribution of spike latency and minimum voltage depolarizing rate following relief from hyperpolarization of Magno (red) and Parvo OXT (green) neurons. Blue dashed line indicates the boundary between Magno and Parvo

647, 1:800, Invitrogen) in PBS for 2 h at RT, then rinsed with PBS for 3 times and mounted onto slides, dried, and covered under glycerol:TBS (3:1) with Hoechst 33342 (1:1000, Thermo Fisher Scientific). Sections were imaged with an Olympus VS120 slide scanning microscope. Confocal images were acquired with a Nikon A1 confocal laser scanning microscope with 25× objectives. Images were analyzed in ImageJ (Fiji).

Electrophysiological data analyses. Electrophysiological data would be collected when neurons had a persistent stable activity in 3 min after break-in. Three protocols were applied to record neuronal spontaneous activity, current injection induced firing, and response after relief from hyperpolarization, respectively (Fig. 1B,D). Electrophysiological data were analyzed following previous studies (Stern, 2001; Ekins et al., 2020; W. Zhang et al., 2021). For Protocol 1, spontaneous firings were observed in most of recorded neurons, excepting only three quiescent neurons (3 in 224 neurons). Neuronal activities recorded in this protocol were used to analyze neuronal spontaneous firing rate (sFreq), coefficient of variation (CV) of interspike interval (ISI), and all the spike properties. For phasic firing neurons (also called bursting), intraburst segment was selected for calculating CV and spike properties.

For analyzing spike properties, neuronal spikes were detected and aligned at spike peak time and averaged. The 100 ms data around spike peak of averaged spike were taken for phase plot analysis (membrane potential V_m vs dV_m/dt). According to averaged spike and phase plot analysis, the following parameters were calculated: spike peak (Peak, the highest membrane potential), spike threshold (Thre, the membrane potential of the first time dV_m/dt reaching 5 mV/ms), spike amplitude (Amp, the subtraction between spike peak and spike threshold), half-width (HW, the duration between membrane potential shoot over and drop below half amplitude), depolarizing rate max (DepoR, the maximum value of phase plot on y axis), repolarizing rate max (RepoR, the minimum value of phase plot on y axis), spike rise time (RisT, time between 10% and 90% of spike amplitude on depolarizing phase), decay time (DecT, time between 10% and 90% of spike amplitude on repolarizing phase), after-hyperpolarization (AHP) amplitude (AHPAmp, membrane potential difference between spike threshold and the lowest potential in the hyperpolarization phase), AHP latency (AHPLat, the time from spike threshold to the lowest potential of spike), depolarization after hyperpolarization (ADP) occurrence (ADPOcc, the probability of ADP occurred during spontaneous firing), and ADP amplitude (ADPAmp, the membrane potential difference between AHP trough and ADP peak).

For Protocol 2, currents were injected into neurons with 10 pA increments and 250 ms interval, and current injected in the first sweep was -100 pA. As neurons have spontaneous activity, averaged membrane potential in the steady state with -20 pA current injected was approximately considered as resting membrane potential (RMP, mV). Input resistance (R_{in} , $M\Omega$) was determined according to averaged membrane

potential in the steady state of -20 and -100 pA. The initial 30 ms voltage response with -100 pA current injection was fitted with a single exponential curve, and membrane time constant (τ , ms) was obtained. Membrane capacitance (Capa, pF) was calculated by $Capa = \tau/R_{in}$.

For Protocol 3, neurons were first injected with a negative current (based on the results from Protocol 2) to make membrane potential hyperpolarize to -100 mV for 250 ms, and then repolarized with a 250 ms current injection from -30 to 65 pA with 5 pA increments. The latency from hyperpolarization relief to the time of the earliest spike fired among all sweeps was defined as the spike latency (SpkL, ms). The first and second ISIs after relief from hyperpolarization were defined as interval1 (ISI1, ms) and interval2 (ISI2), respectively. Adaption index (AdpI) was defined as $(ISI1 - ISI2)/ISI1$.

Morphologic data analyses. After electrophysiological recording, acute slices were fixed in 4% PFA at 4°C for at least 24 h, and subsequently were immunostained with AlexaFluor-488 streptavidin (1:800, 016-540-084, Jackson ImmunoResearch Laboratories) to visualize the morphology of recorded neurons. The confocal and VS120 images were used for morphology reconstruction and spatial location identification, respectively (Fig. 1B–D). Neuronal processes and soma were manually reconstructed using Neurolucida (MBF Bioscience), and morphologic features were extracted and analyzed by Neurolucida Explorer (MBF Bioscience). A total of seven morphologic parameters were measured (Sholl, 1953; Li et al., 2018; Ekins et al., 2020) (Fig. 1B,D), including soma area (SomaA, the area of cell body after projection along z axis), primary dendrite number (DenN, the number of dendrites originating from the cell body), node number (NodeN), dendritic ending number (EndN), total dendritic length (TDL), mean dendritic length (MDL, the average length of every primary dendrites), and process area (also called convex hull area, HullA, the area of convex hull of dendrites after z projection). Sholl analysis measured the intersections between a series of soma center circles with $10 \mu m$ radius increments and neuronal dendrites. The polar histogram was defined as the normalized dendritic length in every direction, and was used to analyze the distribution of dendrites in different directions.

To characterize the spatial location of recorded OXT neurons in PVN, serial $50\text{-}\mu m$ -thick slices of whole PVN from Oxt-Cre; Ai3 mouse strain were imaged with Olympus VS120 slide scanning microscope as the atlas of PVN OXT neurons. In combination with the confocal images, microscope images of biocytin-labeled neurons, the atlas of PVN OXT neurons, and the Paxinos adult mouse brain atlas, neurons recorded in this study were manually mapped onto six rostral–caudal parts of PVN (-0.58 , -0.70 , -0.82 , -0.94 , -1.06 , and -1.22 mm away from bregma), and the distribution of OXT neurons in seven PVN subdivisions (PaAP, PaMM, PaV, PaLM, PaDC, PaMP, and PaPo) was determined according to the Paxinos adult mouse brain atlas.

Correlation and clustering analyses. The 29 morpho-electric parameters we calculated as described above were used for analyzing the correlations between different parameters and neuronal clustering. Neurons were excluded if any of morpho-electric parameters was lacked, and 203 of 224 neurons were included for correlation and clustering analyses. Pearson correlation coefficient between every two parameters were calculated. The Pearson correlation coefficient matrix was demonstrated by a heatmap, and significant level of correlation was indicated by circle size; level of significance <0.05 was reported.

All morpho-electric properties were z -scored to center data and eliminate the difference of scale before clustering. Unsupervised hierarchical clustering was based on cosine distances and the average method. z -scored data were used to plot heatmap, and distance matrix was used to compute dendrogram in MATLAB (The MathWorks). A total of 203 of 224 neurons were included in the clustering analysis, and classified into three main types with the hierarchical clustering trees cutoff at the height of 0.9 (the largest cluster distance is 1.2). Each main type was further divided into two subtypes. The two-dimensional Uniform Manifold Approximation and Projection plot based on the principal components of morpho-electric properties was used to visualize the distribution of different subtypes (Becht et al., 2019).

Quantification and statistical analysis. All electrophysiological data analyses were performed using MATLAB (The MathWorks), pClamp10

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neurons. Insets, Example traces of a Parvo neuron (top) with a long spike latency and a Magno neuron (bottom) with a short spike latency. **C**, Left, Summary about the shortest latency to spike following relief from hyperpolarization for Magno and Parvo neurons. $n = 156$ Magno neurons, and $n = 68$ Parvo neurons. Right, Distribution of spike latency from all PVN OXT neurons and ksdensity fitting of the distribution. **D**, Summary data about the first ISI (Interval1 or ISI1, bottom left) and adaption index (bottom right) of repolarization-induced spikes. **E**, Example traces of irregular firing, regular firing, and phasic firing. **F**, Summary about the CV of spontaneous activity in Magno and Parvo neurons. **G–K**, Same as in **F**, but for spontaneous firing rate (**G**), resting membrane potential (**H**), membrane time constant (**I**), input resistance (**J**), and membrane capacitance (**K**). **L**, Spike number change with different current injections. **M**, Examples of averaged action potential (left) and voltage change phase (right) of Magno (top) and Parvo (bottom) neurons. Bottom left, Inset, A prominent ADP. **N**, Summary about spike threshold in Magno and Parvo neurons. **O–V**, Same as in **N**, but for spike amplitude (**O**), spike peak (**P**), rise time and decay time (**Q**), depolarizing rate maximum (**R**), repolarizing rate maximum (**S**), spike half-width (**T**), AHP amplitude and latency (**U**), and ADP amplitude and occurrence probability (**V**). $n = 151$ – 158 Magno neurons and $n = 67$ – 68 Parvo neurons for each electrophysiological parameter. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Mann–Whitney test. Horizontal lines in violin plot indicate quartiles and median.

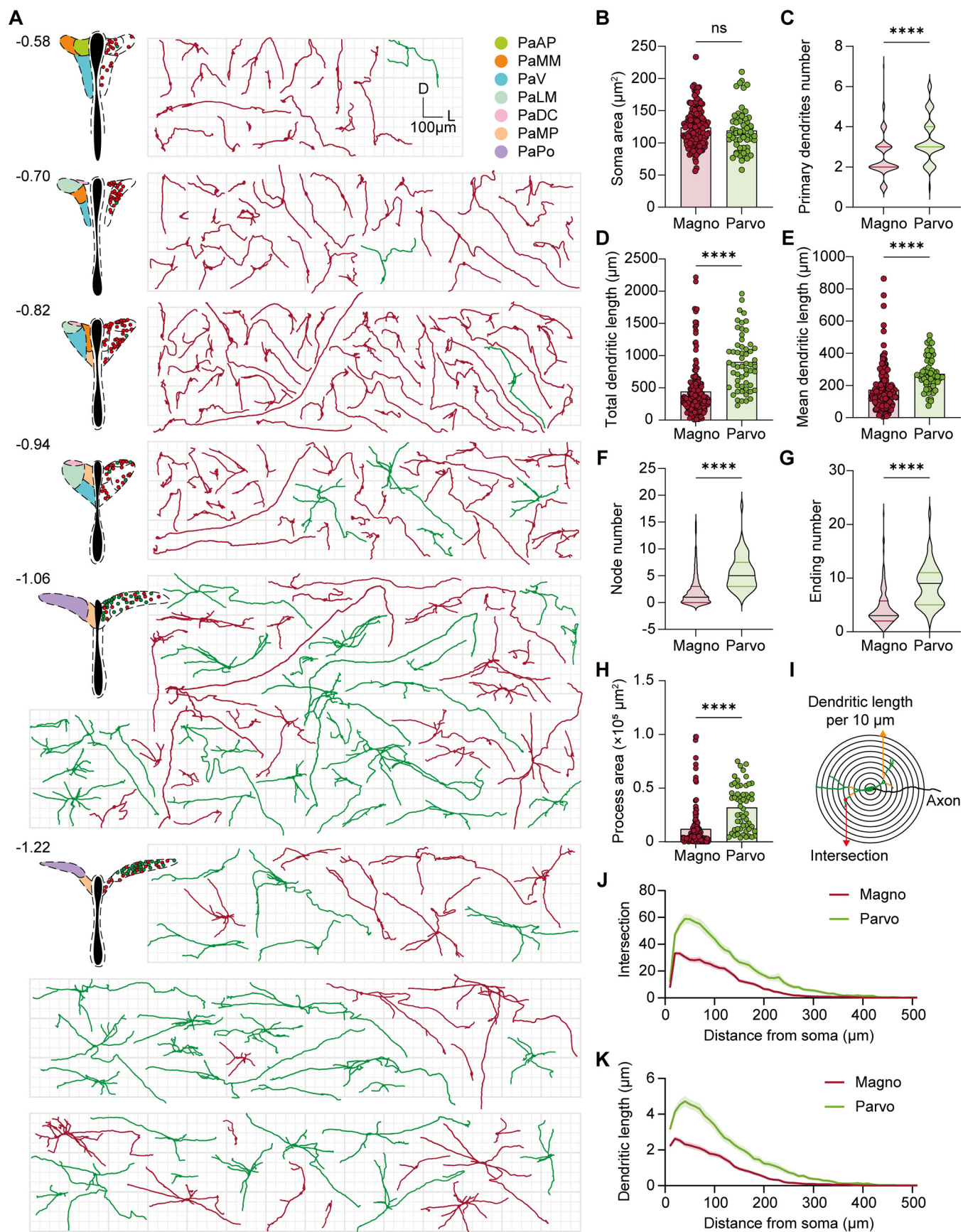


Figure 3. Morphological properties of PVN OXT Magno and Parvo neurons. **A**, The reconstructed morphologies and spatial distribution of 156 Magno (red) and 57 Parvo (green) neurons in different PVN subregions. D, Dorsal; L, lateral to third ventricle. Left number, distance to bregma (mm). **B**, Soma area of Magno and Parvo OXT neurons. **C–H**, Same as in **B**, but for primary dendrite number (**C**), total dendritic length (**D**), mean dendritic length (**E**), branch nodes number (**F**), branch ending number (**G**), and process area (**H**). **I**, A Sholl analysis example. **J**, **K**, Sholl

Table 1. Physiologic and morphologic properties of PVN magno OXT neurons are largely similar in male and female mice

	Female (<i>N</i> = 13 mice)	Male (<i>N</i> = 10 mice)	<i>n</i> (F, M)	<i>p</i>
Electrophysiology				
sFreq (Hz)	7.025 ± 0.4322	5.959 ± 0.4040	86, 69	0.1884
Spike amplitude (Amp, mV)	53.64 ± 1.008	54.10 ± 0.9803	84, 68	0.8491
Spike peak (Peak, mV)	25.13 ± 0.8503	24.22 ± 0.8233	84, 68	0.2978
Rise time (RisT, ms)	0.8687 ± 0.02726	0.8985 ± 0.03608	83, 68	0.7417
Decay time (DecT, ms)	1.928 ± 0.07106	1.910 ± 0.08454	83, 68	0.7613
Depolarizing rate maximum (DepoR, mV/ms)	69.40 ± 3.001	66.98 ± 3.429	84, 68	0.4654
Repolarizing rate maximum (RepoR, mV/ms)	−28.52 ± 1.446	−29.07 ± 1.625	84, 68	0.8404
Half-width (HW, ms)	2.190 ± 0.06667	2.199 ± 0.08054	84, 68	0.9609
Spike threshold (Thre, mV)	−29.18 ± 0.4159	−29.88 ± 0.3957	84, 68	0.3118
Resting membrane potential (RMP, mV)	−61.03 ± 1.193	−64.48 ± 1.278	87, 69	0.0908
Input resistance (Rin, MΩ)	962.6 ± 33.34	967.4 ± 39.14	87, 69	0.9404
Membrane time constant (Tau, ms)	29.36 ± 1.250	30.28 ± 1.143	87, 69	0.4765
Membrane capacitance (Capa, pF)	33.33 ± 1.703	33.85 ± 1.658	87, 69	0.4377
Spike latency (SpkL, ms)	84.04 ± 3.348	82.29 ± 4.038	87, 69	0.4683
Interval1 (ISI1, ms)	40.33 ± 1.332	36.85 ± 1.130	86, 67	0.0630
Adaption index (Adpl)	0.08965 ± 0.01170	0.05192 ± 0.01625	82, 64	0.1422
AHP latency (AHPLat, ms)	18.45 ± 0.8213	18.12 ± 1.051	85, 69	0.8033
AHP amplitude (AHPAmp, mV)	18.59 ± 0.4939	18.04 ± 0.6212	85, 69	0.5165
ADP occurrence (ADPOcc)	0.07814 ± 0.01815	0.1079 ± 0.02807	84, 69	0.7818
ADP amplitude (ADPAmp, mV)	1.135 ± 0.1168	1.064 ± 0.1096	84, 69	0.3864
Bursting (Burst)	0	2	84, 69	—
CV	0.4597 ± 0.03834	0.5249 ± 0.05383	84, 68	0.4906
Morphology				
Soma area (SomaA, μm ²)	121.4 ± 3.031	127.9 ± 3.757	87, 69	0.2777
Primary dendrite number (DenN)	2.437 ± 0.1081	2.507 ± 0.1161	87, 69	0.5594
Node number (NodeN)	1.954 ± 0.2988	2.580 ± 0.3476	87, 69	0.0391*
Ending number (EndN)	4.195 ± 0.3563	4.797 ± 0.4075	87, 69	0.1416
Process area (Hulla, μm ²)	11709 ± 2002	15421 ± 3676	87, 69	0.3399
Total dendritic length (TDL, μm)	424.9 ± 43.50	471.0 ± 50.85	87, 69	0.4216
Mean dendritic length (MDL, μm)	166.5 ± 13.81	182.9 ± 16.55	87, 69	0.5108

**p* < 0.05 (Mann–Whitney test).

(Molecular Devices), or GraphPad Prism (GraphPad). Image analyses were conducted in Imaris (Oxford Instruments), NeuroLucida Explorer (MBF Bioscience), and ImageJ (Fiji, National Institutes of Health). Whenever possible, data were analyzed blind to condition. The number of neurons recorded and the number of animals used in every experiment are provided in the figure legends. Group data are expressed as mean ± SEM. Normality was tested using D'Agostino and Pearson omnibus normality test. For two-group comparisons, statistical significance was determined by two-tailed paired or unpaired Student's *t* tests, or Wilcoxon Signed-Rank test or Mann–Whitney test when assumptions for parametric testing were not satisfied. For multiple group comparisons, one-way ANOVA tests were used for normally distributed data, followed by *post hoc* analyses. For data that were not normally distributed, nonparametric tests for the appropriate group types were used instead. *p* < 0.05 was considered statistically significant.

Results

Distinct electrophysiological properties between PVN Magno and Parvo OXT neurons

To specifically investigate the properties of PVN OXT neurons, our experiments were conducted with nonbreeding male and female Oxt-Cre; Ai3 transgenic mice (postnatal 85–97 d). The reliability and efficiency of Oxt-Cre; Ai3 mice were confirmed with OXT immunostaining (Fig. 1A), and we observed that

>86% eYFP⁺ neurons were OXT-immunopositive (86.05%, 870 of 1011 eYFP⁺ neurons from 2 mice) and >89% OXT-immunopositive neurons were eYFP⁺ (89.51%, 870 of 972 OXT-immunopositive neurons from 2 mice) (Fig. 1A). With the eYFP signal to identify PVN OXT neurons, we conducted electrophysiological recording in current-clamp mode, and the physiological and morphologic properties of OXT neurons were measured after recording as described in Materials and Methods (Fig. 1B–D). We totally recorded 235 PVN OXT neurons, and 224 of 235 neurons from 23 mice have good electrophysiological properties and were included for the analyses in this study.

OXT neurons are traditionally classified into Magno and Parvo subtypes based on the transient outward rectification and latency to spike following relief from hyperpolarization (Luther and Tasker, 2000; Eliava et al., 2016; Xiao et al., 2017; Lewis et al., 2020). We defined the minimum depolarizing rate of membrane potential before the first spike generation during depolarization as the rectified depolarizing rate. As shown in Figure 2A, B, PVN OXT neurons are clearly separated into Magno and Parvo subtypes with the rectified depolarizing rate cutoff at ~0.4. In general, Magno neurons have a longer spike latency than Parvo neurons (Magno latency: 83.27 ± 2.58 ms, *n* = 156 neurons; Parvo latency: 23.63 ± 1.01 ms, *n* = 68 neurons. *p* < 0.0001, Mann–Whitney test. Fig. 2C), although a few Magno OXT neurons exhibit a short latency to spike (the proportion of neurons with spike latency < 50 ms is 8.97%, Fig. 2B). The ratio of Magno OXT neurons and Parvo OXT neurons we recorded is 2.29:1, which is consistent with the previous study detected based on Fluoro-Gold tracing (Lewis et al., 2020). In addition to the spike latency, Magno and Parvo OXT neurons have different ISI

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analysis of Magno and Parvo neurons about total number of Sholl intersections (*I*) and dendritic length per 10 μm (*M*). *n* = 156 Magno neurons, and 57 Parvo neurons. *****p* < 0.0001 (Mann–Whitney test). Horizontal lines in violin plot indicate quartiles and median.

Table 2. Physiologic and morphologic properties of PVN parvo OXT neurons are largely similar in male and female mice

	Female (<i>N</i> = 13 mice)	Male (<i>N</i> = 10 mice)	<i>n</i> (F, M)	<i>p</i>
Electrophysiology				
sFreq (Hz)	7.073 ± 0.6706	5.504 ± 0.5919	33, 35	0.0623
Spike amplitude (Amp, mV)	56.42 ± 1.693	57.61 ± 1.165	33, 35	0.9514
Spike peak (Peak, mV)	26.05 ± 1.301	26.77 ± 1.016	33, 35	0.8263
Rise time (RisT, ms)	0.9091 ± 0.05989	0.8114 ± 0.04724	33, 35	0.2934
Decay time (DecT, ms)	1.673 ± 0.1072	1.506 ± 0.07124	33, 35	0.3377
Depolarizing rate maximum (DepoR, mV/ms)	79.48 ± 6.846	84.41 ± 5.883	33, 35	0.5831
Repolarizing rate maximum (RepoR, mV/ms)	−35.64 ± 3.126	−38.54 ± 3.090	33, 35	0.4715
Half-width (HW, ms)	1.967 ± 0.1313	1.771 ± 0.08741	33, 35	0.4285
Spike threshold (Thre, mV)	−30.36 ± 0.6139	−30.84 ± 0.5022	33, 35	0.5747
Resting membrane potential (RMP, mV)	−61.99 ± 1.803	−64.14 ± 1.561	32, 35	0.2274
Input resistance (Rin, MΩ)	618.8 ± 34.92	743.4 ± 36.55	32, 35	0.0125*
Membrane time constant (Tau, ms)	28.81 ± 1.951	30.07 ± 1.352	33, 35	0.2608
Membrane capacitance (Capa, pF)	50.16 ± 3.837	43.06 ± 2.561	32, 35	0.1043
Spike latency (SpkL, ms)	23.03 ± 1.433	24.21 ± 1.443	33, 35	0.6020
Interval1 (ISI1, ms)	81.36 ± 8.645	74.21 ± 6.777	32, 35	0.7153
Adaption index (Adpl)	0.3818 ± 0.05068	0.4131 ± 0.03732	30, 33	0.9836
AHP latency (AHPLat, ms)	17.56 ± 1.786	15.79 ± 1.700	33, 35	0.4696
AHP amplitude (AHPAmp, mV)	16.39 ± 0.5885	16.62 ± 0.7214	33, 35	0.5500
ADP occurrence (ADPOcc)	0.2128 ± 0.05506	0.2490 ± 0.05773	33, 35	0.7145
ADP amplitude (ADPAmp, mV)	1.093 ± 0.1900	1.026 ± 0.1213	33, 35	0.5582
Bursting (Burst)	0	4	33, 35	—
CV	0.3880 ± 0.08731	0.4209 ± 0.07184	33, 35	0.5500
Morphology				
Soma area (SomaA, μm ²)	112.2 ± 4.200	125.7 ± 7.260	28, 31	0.4645
Primary dendrite number (DenN)	3.308 ± 0.2403	3.355 ± 0.1943	26, 31	0.9179
Node number (NodeN)	5.654 ± 0.7337	5.645 ± 0.4942	26, 31	0.7100
Ending number (EndN)	8.500 ± 0.8362	8.581 ± 0.6218	26, 31	0.7401
Process area (Hulla, μm ²)	30712 ± 3731	33102 ± 4069	26, 31	0.7566
Total dendritic length (TDL, μm)	935.4 ± 88.73	873.6 ± 74.71	26, 31	0.6277
Mean dendritic length (MDL, μm)	288.5 ± 20.49	259.3 ± 17.29	26, 31	0.2346

**p* < 0.05 (Mann–Whitney test).

patterns for spikes following relief from hyperpolarization as shown in Figure 2D. Compared with Magno neurons, Parvo OXT neurons have a larger ISI1 and adaptation index (Fig. 2D).

Both spontaneous activity and different current injection-induced activity were recorded and analyzed as shown in Figure 1D. PVN OXT neurons we recorded exhibited four types of spontaneous activity according to CV (Fig. 2E): regular firing, with CV no more than 0.5 (73.66%, 165 in 224 neurons); irregular firing, with CV >0.5 (22.32%, 50 in 224 neurons); phasic firing, with bursting observed during firing (2.68%, 6 in 224 neurons); and another three quiescent neurons. The CV in Magno neurons is significantly larger than that in Parvo neurons (Fig. 2F), which is consistent with more Magno neurons exhibiting irregular firing mode (26.92% for Magno neurons, 42 in 156 neurons; 11.76% for Parvo neurons, 8 in 68 neurons). Although spontaneous firing rate, resting membrane potential, and membrane time constant are similar between Magno and Parvo neurons (Fig. 2G–I), Magno OXT neurons have a higher input resistance and a lower membrane capacitance than Parvo neurons (Fig. 2J,K). In line with the higher input resistance, positive current injection induced more action potentials in Magno OXT neurons than Parvo neurons (Fig. 2L).

Since the expression levels of potassium and calcium-related channels in Magno and Parvo neurons are different (Lewis et al., 2020), and voltage-gated ion channel expressions determine action potential properties, we analyzed the differences of spike properties between Magno and Parvo neurons. Only neurons with spontaneous firing activity were included for analyzing spike properties, and 12 spike-related parameters were calculated (Fig. 2M; see Materials and Methods). Spike threshold is similar

between Magno neurons and Parvo OXT neurons (Fig. 2N), but Parvo neurons have a significantly larger spike amplitude than Magno neurons (Fig. 2O). Although spike rise time and spike peak values do not show significant distinctions between Magno and Parvo neurons, the Magno neurons have a longer spike decay time (Fig. 2P,Q). Meanwhile, Magno neurons have smaller maximum depolarizing rate and repolarizing rate (Fig. 2R,S), which are consistent with a larger spike half-width observed in Magno neurons (Fig. 2T). AHP occurs because some delayed rectifiers are still open and the membrane is therefore more permeable to potassium. For PVN OXT neurons, Magno subtype has a larger AHP than Parvo neurons (Fig. 2U). An after-depolarization (ADP) after the termination of a single spike, which is related to the T-type calcium channel (Jung et al., 2001; Deleuze et al., 2012), was observed in some OXT neurons (Fig. 2M). Parvo neurons have a higher probability to generate an ADP following an action potential, but the amplitude of ADP in Magno and Parvo neurons is not significantly different (Fig. 2V).

Parvo OXT neurons have more complicated morphology than Magno neurons

Since biocytin was loaded into OXT neurons during electrophysiological recording, morphology properties of PVN OXT neurons were analyzed. Only the neurons with good break-in and electrophysiological properties were considered for morphology analyses. Neuronal morphologies were reconstructed in Neurolucida based on a digitized image stack after staining and imaging, and seven morphology properties, Sholl analysis, and polar histogram were measured with Neurolucida explorer as shown in Figure 1 (see Materials and Methods). A total of 213 of 224 neurons from 23

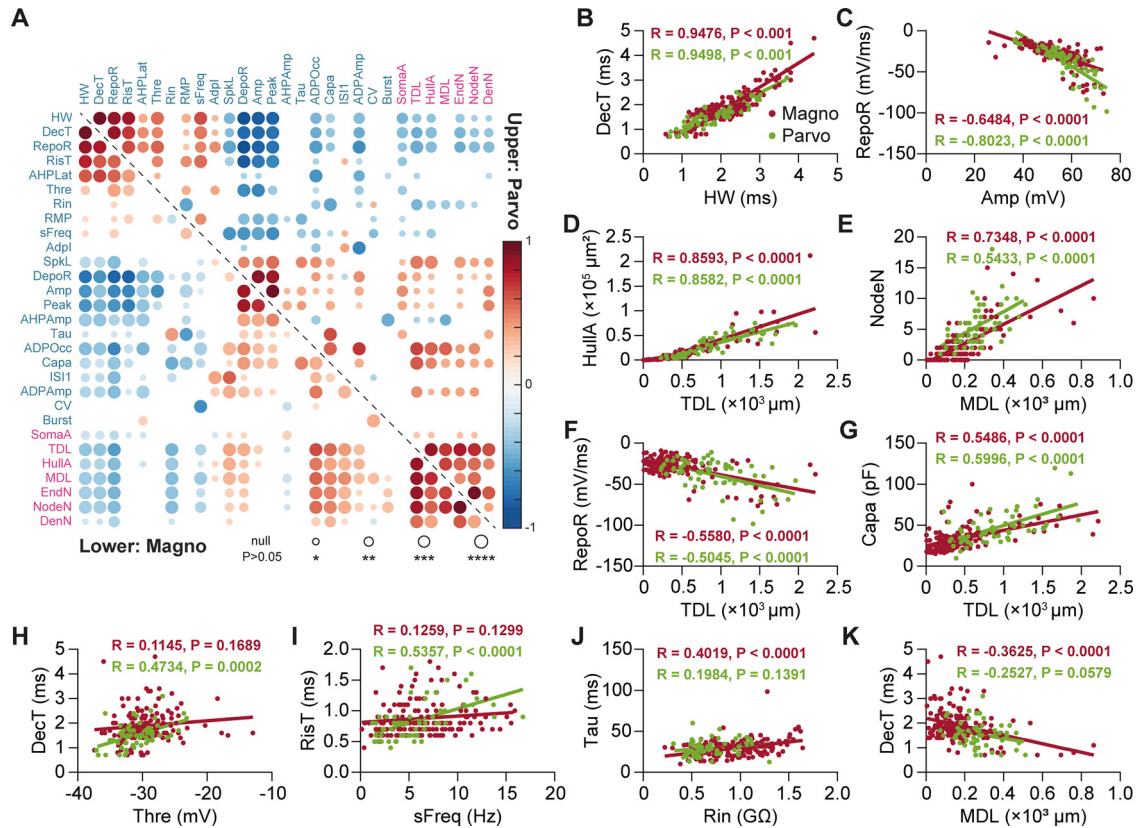


Figure 4. Correlation between morpho-electric properties in PVN OXT Magno and Parvo neurons. **A**, Correlation index (labeled by colormap) and significant level (labeled by circle size) between every pair of morpho-electric properties in Magno (bottom left) and Parvo neurons (top right). **B**, Scatter plot of spike half-width and spike decay time from every Magno (red circles) and Parvo (green circles) neurons. The relation between half-width (HW) and decay time (DecT) was linearly fitted for Magno (red line) and Parvo (green line) neurons, separately. **C–K**, Same as in **B**, but for the relation between spike amplitude (Amp) and repolarizing rate maximum (RepoR) (**C**), total dendritic length (TDL) and convex hull of 2D process area (HullA) (**D**), mean dendritic length (MDL) and dendritic node number (NodeN) (**E**), TDL and repolarizing rate maximum (RepoR) (**F**), TDL and membrane capacitance (Capa) (**G**), spike threshold (Thre) and DecT (**H**), sFreq and spike rise time (RisT) (**I**), input resistance (Rin) and membrane time constant (Tau) (**J**), and MDL and DecT (**K**). $n = 146$ Magno neurons, and $n = 57$ Parvo neurons. Pearson's coefficient correlation two-sided. Terms: half-width (HW), decay time (DecT), repolarizing rate maximum (RepoR), rise time (RisT), AHP latency (AHPLat), spike threshold (Thre), input resistance (Rin), resting membrane potential (RMP), sFreq, adaption index (Adpl), spike latency (SpkL), depolarizing rate maximum (DepoR), spike amplitude (Amp), spike peak (Peak), AHP amplitude (AHPAmp), membrane time constant (Tau), ADP occurrence (ADPOcc), membrane capacitance (Capa), interval1 (ISI1), ADP amplitude (ADPAmp), coefficient of variation (CV), bursting (Burst), soma area (SomaA), total dendritic length (TDL), process area (HullA), mean dendritic length (MDL), ending number (EndN), node number (NodeN), and primary dendrite number (DenN).

mice (156 Magno neurons and 57 Parvo neurons) were included in the morphology analyses, and 11 of 224 neuronal morphologies were destroyed or not found after electrophysiological recording. Reconstructed morphology and spatial distribution of OXT neurons we recorded are shown in Figure 3A.

Although previous studies suggested that Magno OXT neurons in rats have larger soma than Parvo OXT neurons (Eliava et al., 2016), we did not observe significant difference in soma area between these two types of neurons (Fig. 3B). However, we found that Parvo OXT neurons have more primary dendrites (Fig. 3C), longer total and mean dendritic length (Fig. 3D,E), more dendritic nodes (Fig. 3F), more dendritic endings (Fig. 3G), and more process area (Fig. 3H) than Magno neurons. Consistently, in the Sholl analysis (Fig. 3I), more intersections and longer dendritic length per 10 μm window from soma were observed in Parvo neurons (Fig. 3J,K). Together, these results indicate that, compared with Magno neurons, the morphology of Parvo OXT neurons is more complex.

As both male and female mice were used in this study, we separately investigated the gender difference of electrophysiological and morphologic features of PVN Magno (Table 1) and Parvo (Table 2) OXT neurons. For both Magno and Parvo OXT neurons, no significant differences were observed between male

and female in most of features, except that dendritic branch node was more observed in male Magno neurons (Table 1) and male Parvo OXT neurons have significantly larger input resistance (Table 2). Meanwhile, the relative proportion of Parvo and Magno OXT neurons we randomly recorded in this study is higher in males (1:1.97) than in females (1:2.64). Therefore, although PVN Magno and Parvo OXT neurons exhibit sex difference in sporadic morpho-electrical properties, most features of PVN OXT neurons are largely similar between male and female mice.

Morpho-electric properties are correlated in Magno and Parvo neurons

As shown in Figures 2 and 3, we totally analyzed 29 morpho-electric features of PVN OXT neurons. We further investigated whether different neuronal properties are correlated, especially between morphologic and physiological properties. The paired correlations between morpho-electric features in Magno and Parvo OXT neurons were separately calculated, and the results are shown in Figure 4A.

Similar correlation patterns were exhibited in the Magno and Parvo subtypes (Fig. 4A). Strong positive or negative correlations were observed within physiological properties or morphologic properties, including spike half-width versus spike decay time

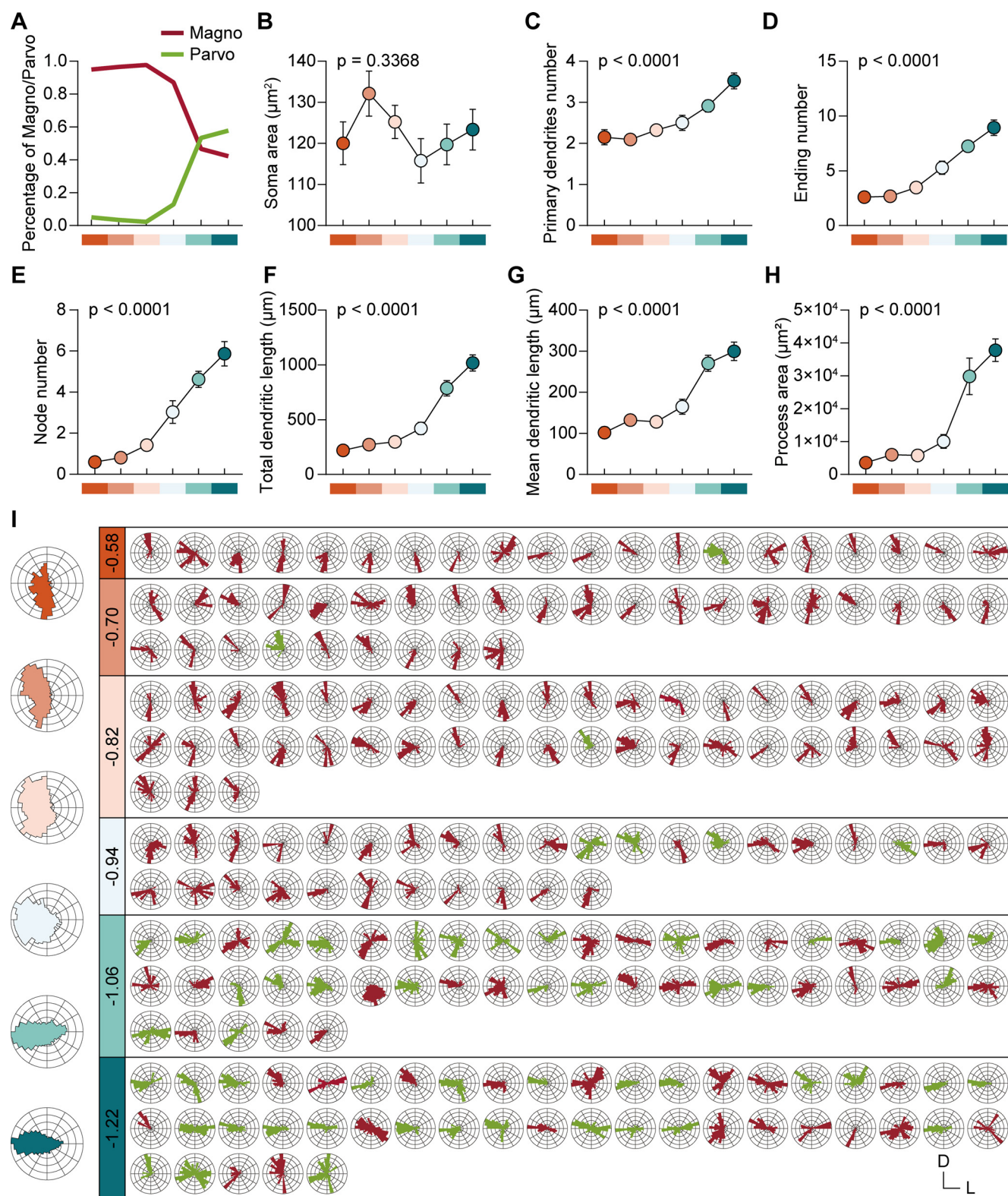


Figure 5. The morphologic properties change in PVN OXT neurons along the rostral–caudal axis. **A**, Proportion of Magno and Parvo OXT neurons at each bregma level we recorded along the rostral–caudal axis. Different colors below from left to right represent -0.58 , -0.70 , -0.82 , -0.94 , -1.06 , and -1.22 mm away from bregma. **B–H**, Same as in **A**, but for soma size (**B**), primary dendrite number (**C**), dendritic endings (**D**), branch nodes (**E**), total and mean dendritic length (**F,G**), and process area (**H**) change along the rostral–caudal axis. $n = 213$ neurons. Kruskal–Wallis test. **I**, Polar histograms of the OXT neurons recorded in different rostral–caudal parts of PVN. Left figures, Polarization summary in different PVN parts. D, Dorsal; L, lateral. $n = 213$ neurons.

(Fig. 4B), spike amplitude versus spike maximum repolarization rate (Fig. 4C), total dendritic length versus process area (Fig. 4D), and mean dendritic length versus dendritic node number (Fig. 4E). In addition, significant correlation was also observed

between some physiological properties and morphologic properties, such as total dendritic length versus repolarization rate maximum (Fig. 4F) and total dendritic length versus membrane capacitance (Fig. 4G).

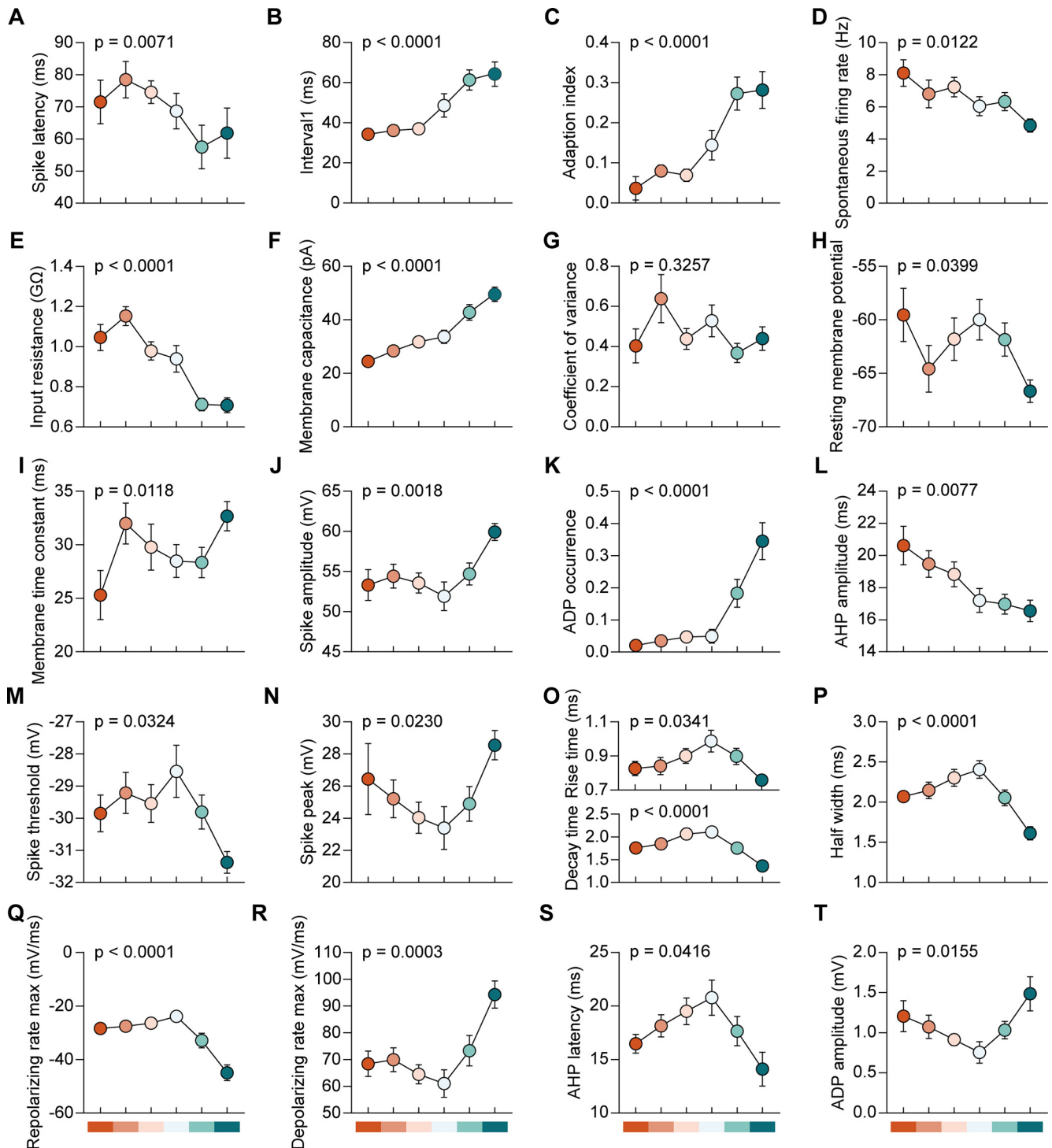


Figure 6. The physiological properties change in PVN OXT neurons along the rostral–caudal axis. **A**, Distribution spike latency of PVN OXT neurons along the rostral–caudal axis. Different colors below from left to right represent -0.58 , -0.70 , -0.82 , -0.94 , -1.06 , and -1.22 mm away from bregma. **B–T**, Same as in **A**, but for the interval1 (**B**) and adaptation index (**C**) of repolarization-induced spikes, spontaneous firing rate (**D**), input resistance (**E**), membrane capacitance (**F**), CV (**G**), resting membrane potential (**H**), membrane time constant (**I**), spike amplitude (**J**), probability of ADP occurrence (**K**), AHP amplitude (**L**), spike threshold (**M**), spike peak (**N**), spike rise time and decay time (**O**), half-width (**P**), maximum spike depolarizing rate (**Q**) and spike repolarizing rate (**R**), spike AHP latency (**S**), and ADP amplitude (**T**). $n = 204$ – 213 neurons for each parameter. Kruskal–Wallis test. Error bars indicate SEM.

Meanwhile, although most morpho-electric features exhibited similar correlations in Magno and Parvo neurons (Fig. 4A), significant correlations between some properties were only observed in either Magno neurons or Parvo neurons. For example, correlations between spike threshold

and spike decay time (Fig. 4H), spontaneous firing rate and spike rise time (Fig. 4I), are only significant in Parvo OXT neurons. However, the correlations between input resistance and membrane time constant (Fig. 4J), mean dendritic length and decay time (Fig. 4K), are only significant in Magno OXT neurons.

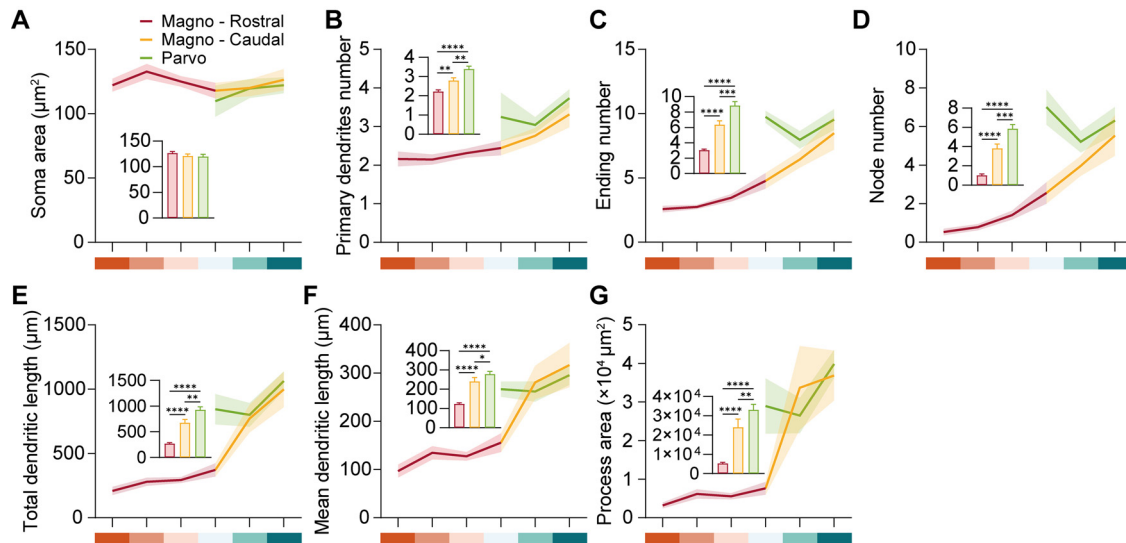


Figure 7. OXT Magno neurons in PVN rostral and caudal regions exhibit distinguishing morphologic properties. **A**, Soma size between rostral Magno (red), caudal Magno (yellow), and Parvo (green) OXT neurons. Different colors below from left to right represent -0.58 , -0.70 , -0.82 , -0.94 , -1.06 , and -1.22 mm away from bregma. The three PVN parts close to bregma (-0.58 , -0.70 , -0.82 mm) were defined as rostral region, and the other three parts (-0.94 , -1.06 , -1.22 mm) as caudal region. $n = 89$, 67 , and 54 for each group. **B–G**, Same as in **A**, but for the primary dendrite number (**B**), ending number (**C**), node number (**D**), total dendritic length (**E**), mean dendritic length (**F**), and process area (**G**). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Kruskal–Wallis with Dunn’s multiple comparisons test. Error bars indicate SEM.

Change of PVN OXT neuronal properties along the rostral–caudal axis

As the mapping results shown in Figure 3A, morphologies of OXT neurons tend to become more complex along the rostral–caudal axis, so we further investigated the properties of OXT neurons in the rostral–caudal axis. We divided PVN into six rostral–caudal parts according to the mouse brain atlas (Fig. 3A). Consistent with previous study (Lewis et al., 2020), more Magno neurons we recorded are distributed in the PVN rostral division, while most Parvo neurons are localized in the PVN caudal division (Fig. 5A), but the soma size of PVN OXT neurons does not show significant change along the rostral–caudal axis (Fig. 5B). Morphologic properties, including primary dendrite number (Fig. 5C), dendritic endings (Fig. 5D), branch nodes (Fig. 5E), total and mean dendritic length (Fig. 5F,G), and process area (Fig. 5H), are monotonically increased along the rostral–caudal axis, which confirm the morphology of PVN OXT neurons becomes more complex. Since PVN is adjacent to the third ventricle, we analyzed the polarization of OXT neurons. As shown in Figure 5I, dendrite distributions in PVN OXT neurons are asymmetric, and most of dendrites radiate toward or along the third ventricle, consistent with previous observation in PVN neurons (van den Pol, 1982). Meanwhile, the neuronal dendrite covering region is becoming narrower along the rostral–caudal axis (Fig. 5I), which suggests that OXT neuronal dendrites are mostly constrained in the PVN region.

The changes of physiological properties along the rostral–caudal axis were also analyzed (Fig. 6). The latency of the first spike following relief from hyperpolarization becomes shorter along the rostral–caudal axis (Fig. 6A), which is consistent with the biased distribution of Magno and Parvo neurons (Fig. 5A). The Interval1 (Fig. 6B) and adaptation index (Fig. 6C) for spikes following relief from hyperpolarization are monotonically increased along the rostral–caudal axis. The spontaneous activity and input resistance are significantly reduced along the rostral–caudal axis (Fig. 6D,E), and membrane capacitance has a reverse change (Fig. 6F), but the CV, resting membrane potential, and membrane time constant do not show monotonical change (Fig.

6G–I). The changes of spike properties exhibit multiple change patterns along the rostral–caudal axis, including spike amplitude and ADP occurrence probability, are monotonically increased (Fig. 6J,K), AHP amplitude is monotonically decreased (Fig. 6L), and spike threshold, spike peak, spike rise time and decay time, spike half-width, maximum depolarizing and repolarizing rates, AHP latency, and ADP amplitude exhibit U-shape or inverted U-shape change (Fig. 6M–T). These results indicate that morphologic properties mainly exhibit monotonical change, similar as the biased distribution of Magno and Parvo neurons in the PVN, but the physiological properties of PVN OXT neurons exhibit various variations along the rostral–caudal axis.

Since morpho-electric properties of PVN OXT neurons exhibit diverse spatial change patterns (Figs. 5 and 6) and Magno neurons are distributed across PVN subregions, we further investigated the changes of Magno neuronal properties across the rostral–caudal axis. We defined the three PVN parts close to bregma (-0.58 , -0.70 , -0.82 mm) as the rostral region, and the other three parts (-0.94 , -1.06 , -1.22 mm) as the caudal region. Only three Parvo neurons we identified are localized in the rostral region (Fig. 3A), so we did not compare the properties of Parvo neurons in rostral and caudal regions. Parvo, rostral Magno, and caudal Magno OXT neurons have similar soma sizes (Fig. 7A). However, the other morphologic features, including primary dendrite number, dendritic node number and ending number, total and mean dendritic length, and process area exhibit significantly different among Parvo OXT neurons, the rostral and caudal Magno neurons (Fig. 7B–G). Compared with the rostral Magno OXT neurons, morphologic properties of caudal Magno neurons are close to the properties of Parvo OXT neurons (Fig. 7).

For physiological properties, several features of the caudal Magno neurons are close to the rostral Magno neurons, including spike latency (Fig. 8A), the Interval1 (Fig. 8B), and adaptation index (Fig. 8C) for spikes following relief from hyperpolarization, CV (Fig. 8G), spike decay time (Fig. 8O), spike half-width (Fig. 8P), and maximum repolarizing rate (Fig. 8R). On the contrary, some physiological properties of

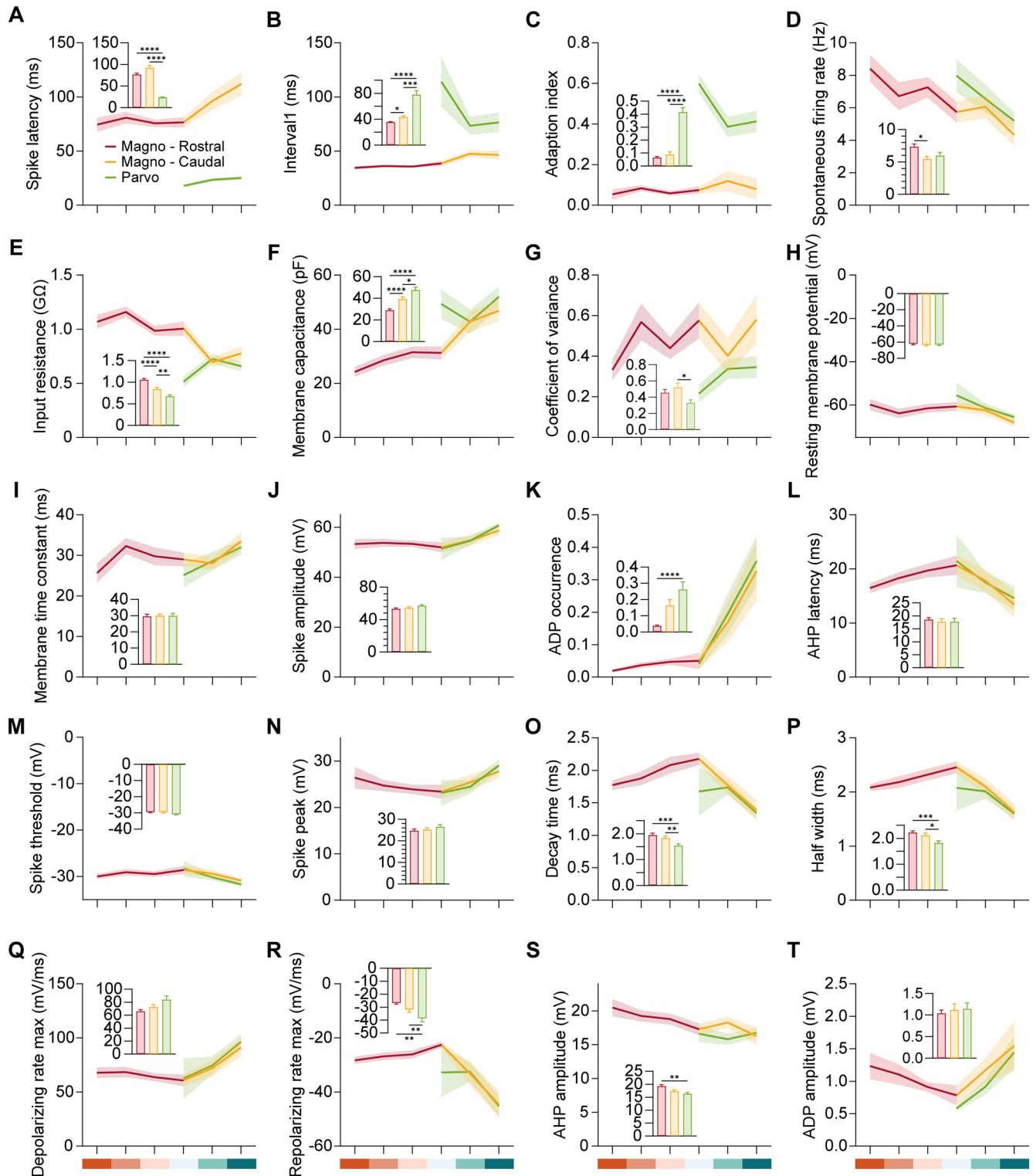
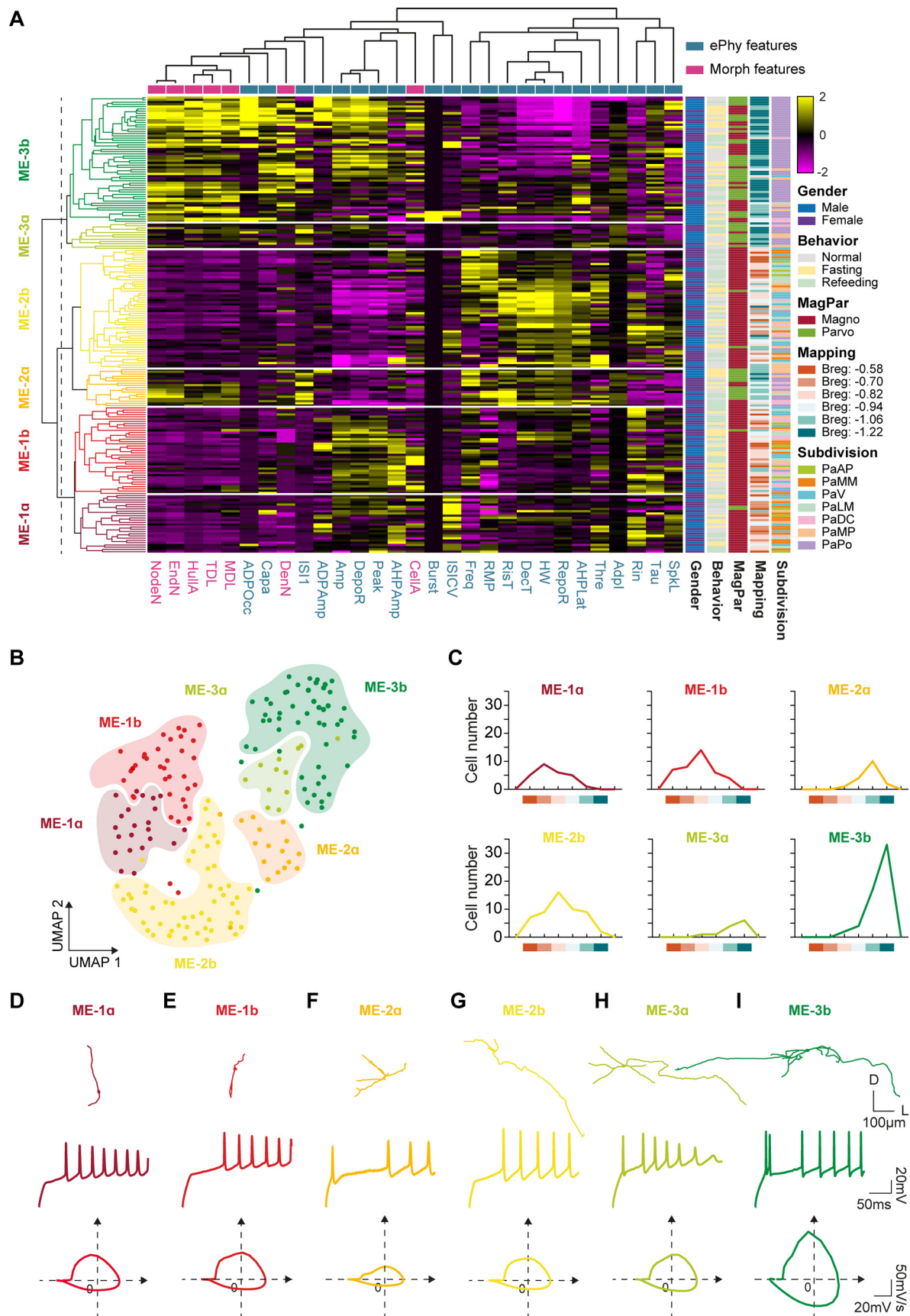


Figure 8. OXT Magno neurons in PVN rostral and caudal regions exhibit distinguishing physiological properties. **A**, Difference of spike latency between rostral Magno (red), caudal Magno (yellow), and Parvo (green) OXT neurons. Different colors below from left to right represent -0.58 , -0.70 , -0.82 , -0.94 , -1.06 , and -1.22 mm away from bregma. The three PVN parts close to bregma (-0.58 , -0.70 , -0.82 mm) were defined as rostral region, and the other three parts (-0.94 , -1.06 , -1.22 mm) as caudal region. $n = 85$, 66 , and 54 for Magno-rostral, Magno-caudal, and Parvo, respectively. **B–T**, Same as in **A**, but for the ISI1 ($n = 85$, 63 , 53 for each group, **B**), adaption index ($n = 83$, 58 , 50 for each group, **C**), spontaneous firing rate, ($n = 85$, 65 , 54 for each group, **D**), input resistance ($n = 85$, 66 , 54 for each group, **E**), membrane capacitance ($n = 85$, 66 , 54 for each group, **F**), CV ($n = 83$, 64 , 54 for each group, **G**), resting membrane potential ($n = 85$, 66 , 54 for each group, **H**), membrane time constant ($n = 85$, 66 , 54 for each group, **I**), spike amplitude ($n = 83$, 64 , 54 for each group, **J**), ADP occurrence ($n = 83$, 65 , 54 for each group, **K**), AHP latency ($n = 83$, 66 , 53 for each group, **L**), spike threshold ($n = 83$, 64 , 54 for each group, **M**), spike peak ($n = 83$, 64 , 54 for each group, **N**), spike decay time ($n = 82$, 64 , 54 for each group, **O**), spike half-width ($n = 83$, 64 , 54 for each group, **P**), spike depolarizing and repolarizing maximum rates ($n = 83$, 64 , 54 for each group, **Q,R**), AHP amplitude ($n = 83$, 66 , 53 for each group, **S**), and ADP amplitude ($n = 83$, 65 , 54 for each group, **T**). $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$; Kruskal–Wallis with Dunn’s multiple comparisons test. Error bars indicate SEM.



caudal Magno neurons are more identical to Parvo neurons, but not to the rostral Magno neurons, including spontaneous firing rate (Fig. 8D), input resistance (Fig. 8E), membrane capacitance (Fig. 8F), ADP occurrence (Fig. 8K), and maximum depolarizing rate (Fig. 8Q). At the same time, other physiological properties of caudal Magno neurons are similar to both Parvo and rostral Magno OXT neurons (Fig. 8), such as resting membrane potential (Fig. 8H), membrane time constant (Fig. 8I), spike amplitude (Fig. 8J), spike threshold (Fig. 8M), spike peak (Fig. 8N), AHP latency and amplitude (Fig. 8L,S), and ADP amplitude (Fig. 8T). Together, these results suggest that rostral and caudal Magno OXT neurons have commonality and individuality, so they should be classified into different subgroups.

PVN OXT neurons are classified into at least six subtypes based on morpho-electric properties

The diverse variations of morpho-electric properties along the rostral–caudal axis as shown in Figures 5–8 suggest that PVN OXT neurons can be classified into more than two types. Hence, total 29 morpho-electric features measured from 203 of 224 PVN neurons in 23 mice were z-scored to standardize the scale and used for the unsupervised classification (see Materials and Methods).

As shown in Figure 9A, B, PVN neurons were categorized into three major types (ME-1, ME-2, and ME-3), and each type was further classified into two subtypes as described in Materials and Methods. ME-1a, ME-1b, and ME-2b are mainly composed of Magno neurons (92.31% in 26 neurons for ME-1a, 97.44% in 39 neurons for ME-1b, 96.23% in 53 neurons for ME-2b); ME-2a and ME-3a are mainly composed of Parvo neurons (70.59% in 17 neurons for ME-2a, 75.00% in 12 neurons for ME-3a); and ME-3b include both Magno and Parvo neurons (55.36% in 56 neurons for ME-3b are Parvo) (Fig. 9A). As shown in Figure 9C, these six subtypes are differently distributed along the rostral–caudal PVN axis. Morphology and electrical response from example neurons in each subgroup are shown in Figure 9D–I. PVN OXT neurons in ME-1a and ME-1b have simple morphology and also similar spike properties, but the CV of spontaneous activity is higher in ME-1a than in ME-1b (Fig. 9A). OXT neurons in ME-2a and ME-2b subgroups share several common physiological properties differing from the other four subgroups, such as higher spontaneous frequency, higher membrane potential, and longer spike rise time and decay time (Fig. 9A). Neurons in ME-2a and ME-2b, respectively, belong to Parvo and Magno neurons, and they have different morphologic properties (Fig. 9A,F,G). ME-3a and ME-3b neurons have similar morphologic properties, but OXT neurons in ME-3b have larger spike amplitude, ADP amplitude, and AHP amplitude (Fig. 9A,H,I). The distribution of different subtypes in PVN subdivision is also investigated, and we observed that most of neurons in ME-3b (83.64%) are located at posterior part of PVN (PaPo); but the other five subtypes contain neurons from multiple PVN subdivisions (Fig. 9A).

Firing activities of ME-3 OXT neurons are changed in different feeding conditions

OXT acts as an anorexigenic factor in controlling food intake, but the effect of PVN OXT neurons on feeding behavior remains

controversial (Sabatier et al., 2013; Romano et al., 2020). We investigated the change of PVN OXT neuronal activity from mice in three feeding states: Normal feeding, 25 h Fasting, and Refeeding 1 h after 24 h fasting (Fig. 1B). Spontaneous activities did not significantly change in different feeding states when considering all the recorded PVN OXT neurons together (FR in Normal: 5.945 ± 0.4037 Hz from 74 neurons; FR in Fasting: 6.195 ± 0.4165 Hz from 68 neurons; FR in Refeeding: 6.994 ± 0.4344 Hz from 80 neurons; $p = 0.1736$, Kruskal–Wallis test). We also did not observe significant firing rate change in different feeding states if PVN OXT neurons were simply divided into Magno and Parvo neurons (Fig. 10A). However, after unsupervised clustering analysis (Fig. 9), the firing activities of ME-3 OXT neurons, but not ME-1 or ME-2 subgroups, were significantly changed in different feeding states (FR in Normal: 4.489 ± 0.4311 Hz from 28 neurons; FR in Fasting: 4.242 ± 0.5020 Hz from 22 neurons; FR in Refeeding: 5.966 ± 0.5645 Hz from 17 neurons; $p < 0.05$, Kruskal–Wallis test), especially the firing rate at refeeding condition was higher than that at fasting condition (Fig. 10B). Since neurons in ME-3 were further classified into ME-3a and ME-3b, only OXT neurons in ME-3b, but not ME-3a, exhibited firing rate change with different feeding conditions (Fig. 10C).

OXT neurons in ME-3 are distributed in the posterior PVN (Fig. 9C), especially in the PaPo subregion ($\sim 79.10\%$ ME-3 neurons in the PaPo; Figs. 9A, 10D,E), and most OXT neurons (80.30% in 66 PaPo OXT neurons) we recorded in the PaPo belong to the ME-3 group. ME-3 neurons can be subdivided into ME-3a and ME-3b (12 neurons for ME-3a, 56 neurons for ME-3b). ME-3b, the only subtype showing activity changing in different feeding conditions, is composed of both Magno and Parvo neurons (25 neurons for Magno, 31 neurons for Parvo) (Fig. 10D,E). We then further compared the morpho-electric properties of ME-3b neurons with other subtypes. ME-3b neurons have more complex morphology than the other five subtypes, including longer total dendritic length (Fig. 10F). ME-3b neurons also show differences in some electrophysiological properties, such as shorter spike decay time (Fig. 10G), higher probability of ADP occurrence (Fig. 10H), and larger membrane capacitance (Fig. 10I). Furthermore, soma size of ME-3b neurons is significantly larger than that of ME-2a and ME-3a, which both are mainly composed of Parvo neurons, while similar to that of ME-1a, ME-1b, and ME-2b, which are mainly composed of Magno neurons (Fig. 10J). Meanwhile, the soma size of Magno and Parvo OXT neurons in ME-3b is similar (Magno: $129.8 \pm 7.33 \mu\text{m}^2$; Parvo: $135.9 \pm 5.95 \mu\text{m}^2$; $n = 31$ and 25 for Magno and Parvo neurons, $p = 0.3967$, Mann–Whitney test), and some Parvo neurons have a large soma area (Fig. 10J). Therefore, OXT neurons in ME-3b are different from the other subtypes, and may be responsible for regulating food intake behavior.

Discussion

OXT is involved in multiple functions and plays pivotal roles in every stage of our life (Lee et al., 2009). Neural function and transcription studies have suggested the diversity of PVN OXT neurons, but the morpho-electric properties and diversity of PVN OXT neurons are not fully investigated. In this study, we systematically characterize PVN OXT neuronal morpho-electric properties and rostral–caudal location distributions, and find that PVN OXT neurons are classified into at least six subtypes according to their morpho-electric properties (Fig. 11). Neither the ensemble activity of PVN OXT neurons nor the activity of

←

PVN OXT neurons along the rostral–caudal axis (labeled with different colors at the bottom). **D**, Morphologic and physiological properties of one example neuron from ME-1a subgroup. **E–I**, Same as in **D**, but for the example neurons from ME-1b (**E**), ME-2a (**F**), ME-2b (**G**), ME-3a (**H**), and ME-3b (**I**) subgroups, respectively. D, Dorsal; L, lateral to third ventricle.

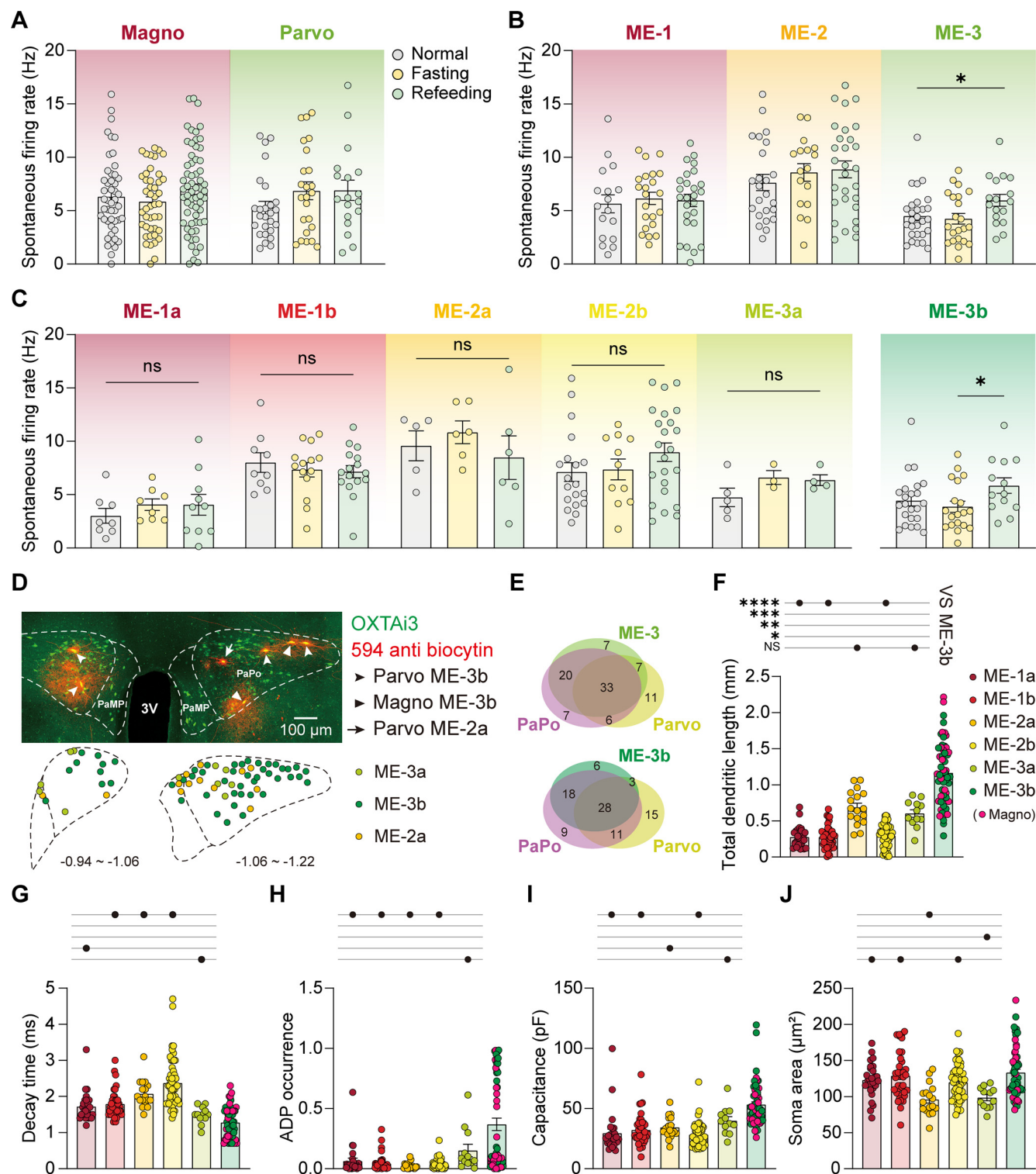


Figure 10. Firing activities of PVN OXT neurons in different feeding states and the properties of ME-3b neurons. **A**, Firing rate of Magno and Parvo neurons in Normal, Fasting, and Refeeding conditions. $n = 48, 44$, and 62 neurons for Normal, Fasting, and Refeeding conditions, respectively, in the Magno group. $n = 26, 24$, and 18 neurons for Normal, Fasting, and Refeeding conditions, respectively, in the Parvo group. **B**, Firing rate of ME-1, ME-2, and ME-3 OXT neurons in Normal, Fasting, and Refeeding conditions. $n = 17, 22$, and 26 neurons for ME-1, $n = 24, 17$, and 29 neurons for ME-2, $n = 28, 22$, and 17 neurons for ME-3 in Normal, Fasting, and Refeeding conditions, respectively. **C**, Firing rate of ME-1a ($n = 8, 8$, and 10 neurons), ME-1b ($n = 9, 14$, and 16 neurons), ME-2a ($n = 5, 6$, and 6 neurons), ME-2b ($n = 19, 11$, and 23 neurons), ME-3a ($n = 5, 3$, and 4 neurons), and ME-3b ($n = 23, 19$, and 13 neurons) OXT neurons in Normal, Fasting, and Refeeding conditions, respectively. * $p < 0.05$ (Mann–Whitney test for Fasting and Refeeding condition). **D**, Top, Example slice of PaPo with neurons labeled by biocytin immunostaining. Bottom, Distribution of ME-3a, ME3b, and ME-2a neurons in the PaPo. **E**, Top, Venn diagram of ME-3, PaPo, and Parvo neurons. Bottom, Venn diagram of ME-3b, PaPo, and Parvo neurons. **F**, Total dendritic length of six subtypes of OXT neurons. Top lines indicate the significant level of difference between ME-3b with the other five subtypes. **G–J**, Same as in **F**, but for spike decay time (**G**), ADP occurrence (**H**), membrane capacitance (**I**), and soma area (**J**). $n = 26, 39, 17, 53, 12$, and 56 neurons for ME-1a, ME-1b, ME-2a, ME-2b, ME-3a, and ME-3b, respectively. Different colors labeled circles in ME-3b subgroup represent Magno (magenta) and Parvo (green) neurons. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$; Kruskal–Wallis test. Error bars indicate SEM.

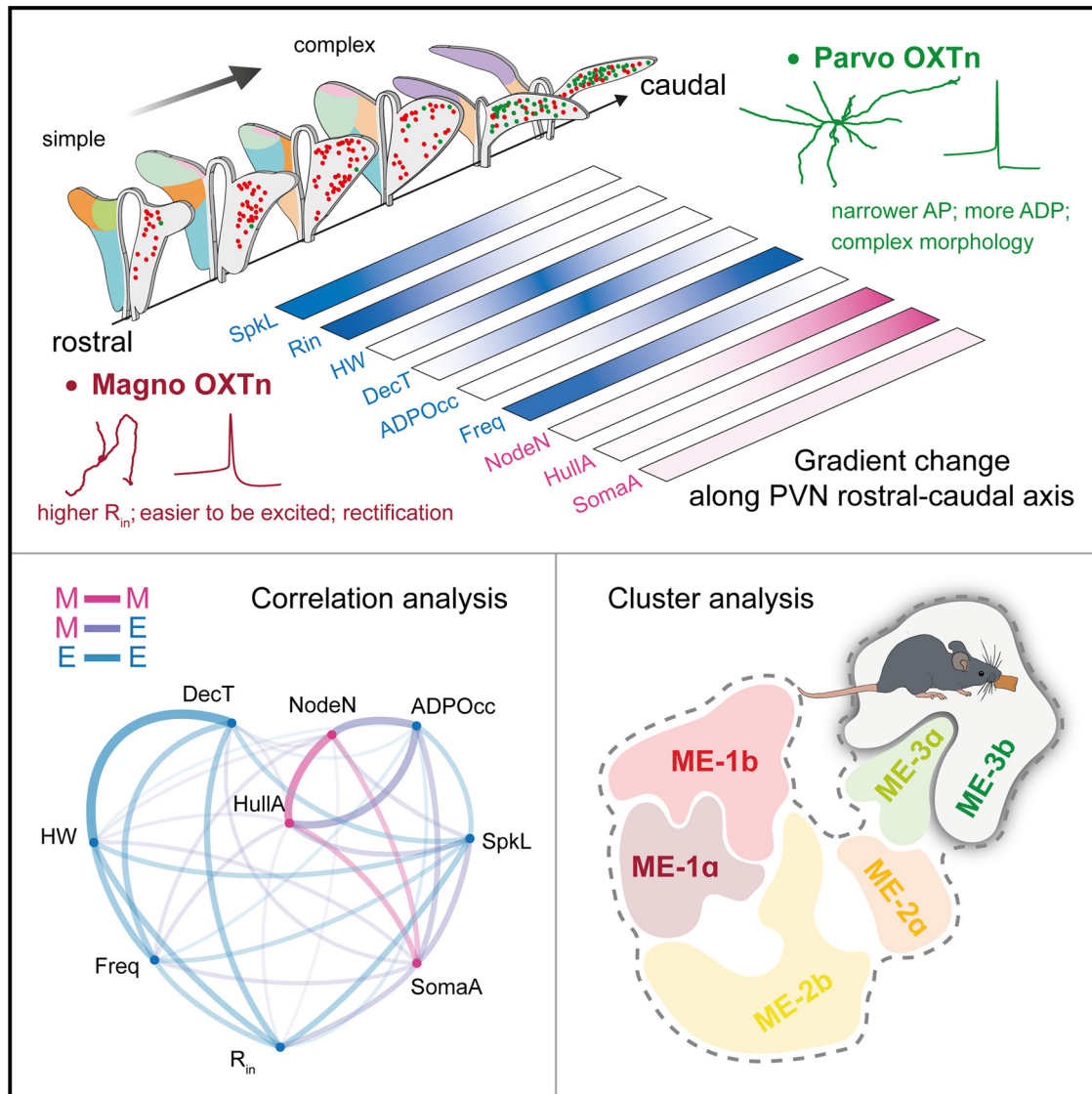


Figure 11. Schematic depiction of the main findings in this study. The main findings include the gradient change of Magno and Parvo OXT neurons along the rostral–caudal axis (top), correlation of morpho-electric features (bottom left), and six subtypes of PVN OXT neurons identified by the unsupervised cluster analyses and the involvement of ME-3b subtype in feeding behavior (bottom right).

Magno and Parvo OXT neurons shows significant difference for mice pretreated in different feeding conditions, but a clustering subgroup with Magno-Parvo mixed neurons in PaPo exhibit significant firing rate change (Fig. 11). Therefore, PVN OXT neurons are diverse, and subtle classification of PVN OXT neurons will advance our investigation about the functions of oxytocinergic systems.

Similar as previous reports about PVN Parvo and Magno neurons (Luther and Tasker, 2000; Stern, 2001; Luther et al., 2002), PVN Parvo and Magno OXT neurons exhibit a lot of distinctions in both physiological and morphologic properties. Although PVN Magno neurons are traditionally suggested to have a larger soma than Parvo neurons, we did not observe obvious difference of soma size of PVN OXT Parvo and Magno neurons in mice. Three possibilities may cause this contradicting result. First, patch-clamp recording may disrupt or distort cell membrane and induce the biased estimation of soma size. During patch recording, neurons could shrink/collapse; and even subtle differences in cell volume could affect subsequent soma size assessment. Second, species differences may also

contribute to this contradicting result. Species differences in PVN organization and OXTergic signal have been reported (Insel et al., 1997; Kádár et al., 2010). Previous studies about soma size of PVN Magno and Parvo neurons were conducted in rats (Sawchenko and Swanson, 1982; Hoffman et al., 1991; Eliava et al., 2016), but few studies directly investigated the soma size difference of these two types in mice. Third, after further clustering classification, we observed that Magno-dominated subgroups, including ME-1a, ME-1b, and ME-2b, have larger soma size than Parvo-dominated ME-2a and ME-3a subgroups, but Parvo OXT neurons in the ME-3b have similar cell body as the Magno neurons (Fig. 10); and this distinctive subgroup may also cause the contradicting result about soma size. Our study finds that PVN Parvo OXT neurons have more dendrites, and PVN Magno OXT neurons have a wider action potential, induced by the longer spike decay time but not spike rise time, which are consistent with the properties of PVN Parvo and Magno neurons (Stern, 2001; Luther et al., 2002; Lewis et al., 2020). The repolarization phase of action potential is mainly determined by the voltage-gated potassium channels, indicating that Magno and Parvo

OXT neurons have distinct voltage-gated potassium channels expression (Luther and Tasker, 2000; Lewis et al., 2020). In rat PVN, Magno and Parvo OXT neurons are suggested to have similar input resistance (Luther and Tasker, 2000), but an electrophysiology study about rat PVN pre-autonomic neurons found that one type of PVN neurons with shorter dendritic length have a significantly higher input resistance (Stern, 2001). In this study, mouse PVN Magno OXT neurons have a larger input resistance, and they will fire more action potentials with positive current injection than Parvo OXT neurons. These indicate that PVN Magno OXT neurons may be easier to be excited to fire more spikes, which is necessary for OXT neurons to release OXT (Wakerley and Lincoln, 1973; Knobloch et al., 2012).

We analyzed the correlation between physiological and morphologic properties of PVN OXT neurons. In addition to strong correlation within physiological properties or morphologic properties, dendritic properties of OXT neurons are also correlated with spike properties and membrane resistance (Fig. 4), suggesting that the morphology of OXT neurons may influence neuronal physiological properties. Neurons integrate synaptic inputs via dendrites and dendritic properties play important roles in regulating neuronal firing patterns and neural excitability (van Elburg and van Ooyen, 2010; Zhu et al., 2016). In hippocampus, minor alterations of dendritic bifurcations of CA1 pyramidal neurons would have effects on the features of action potentials (Ferrante et al., 2013). Neuronal input resistance in PVN neurons was suggested to reflect the total neuronal surface area and membrane sensitivity (Stern, 2001), and PVN OXT neurons also obey this rule. Therefore, more complex dendritic features of Parvo OXT neurons may be one of the factors to induce distinct synaptic integration and spike properties from Magno OXT neurons.

In many brain regions, neuronal properties, including morphology, physiology, and molecular expression, are reported to show gradient change along the anatomic structure (Miliot et al., 2016; Berns et al., 2018; Holley et al., 2018). We found that OXT neurons in PVN are also not homogeneous along the rostral-caudal axis. Similar as previously reported (Lewis et al., 2020), Magno OXT neurons are more distributed in the rostral PVN and Parvo OXT neurons are located at the caudal part. Until now, few studies reported the changes of morpho-electric properties of PVN neurons along the rostral-caudal axis. Our study found that morphologic and electrophysiological features of PVN OXT neurons have diverse change ways along the rostral-caudal axis, including some exhibiting monotonic increase or decrease, some showing U-shape or inverted U-shape change, and several properties displaying no change (Figs. 5 and 6). In addition to the morpho-electric properties, rostral and caudal PVN OXT neurons exhibit different neural circuit projections and functions; that is, rostral PVN OXT neurons project more to the forebrain to regulate social-related behavior and locomotion (B. Zhang et al., 2021), and caudal neurons have more projections to the hindbrain and brainstem to control feeding and nociception (Blevins et al., 2004; Eliava et al., 2016). Molecular expression is required to screen to understand the mechanisms underlying these diverse properties changes along the rostral-caudal axis.

The role of PVN OXT neurons in regulating food intake is controversial (Atasoy et al., 2012; Wu et al., 2012; Sabatier et al., 2013; Sutton et al., 2014). In this study, we directly recorded the spontaneous activity of PVN OXT neurons when mice were pre-treated in different feeding conditions. Unexpectedly, we did not

observe significant difference when considering PVN OXT neurons as a whole, or simply classified into Magno and Parvo neurons, but one subtype of PVN OXT neurons ME-3b, identified by the unsupervised clustering and mainly distributed in the PaPo subregion, is relevant with feeding behavior. The firing rate of ME-3b was increased after refeeding, indicating that more OXT is released to terminate feeding, which is consistent with the anorexic role of OXT (Onaka and Takayanagi, 2019). Modulating PVN OXT neurons alone did not significantly change the food intake behavior (Atasoy et al., 2012; Sutton et al., 2014), but OXT neurons in the posterior PVN (PaPo subregion) projecting to the NTS are suggested to regulate energy homeostasis (Uchoa et al., 2009; Ong et al., 2017). Previous tracing studies found that rat neurons in PaPo region project more to the brainstem and spinal cord (Swanson and Kuypers, 1980; Sawchenko and Swanson, 1982). Combining retrograde circuit tracing and chemogenetic/optogenetic tools may specifically target the NTS-projecting OXT neurons in the PaPo to investigate the function of ME-3b neurons in feeding behavior. Recent studies in rats showed that PVN Parvo OXT neurons project to both the Magno OXT neurons in the SON and neurons in the deep layers of the spinal cord to encode contextual fear memory and promote analgesia (Eliava et al., 2016; Hasan et al., 2019), and social touch information converges to Parvo OXT neurons and then Parvo OXT neurons communicate with Magno OXT neurons to support motivated social communication (Tang et al., 2020). Magno and Parvo OXT neurons in ME-3b may also coordinate with each other to regulate social and pain-related behaviors. Hence, it is necessary to redefine subtypes of PVN OXT neurons to sophisticatedly investigate the functions of OXTergic system (Althammer and Grinevich, 2017).

In this study, we investigated the diversity of OXT neurons only based on the morpho-electric properties but did not screen their gene expression. In recent years, several studies uncovered the diversity of OXT neurons by PVN single-cell RNA sequencing, and at least four subtypes of OXT neurons have been identified (Romanov et al., 2017); however, they did not have neuronal morpho-electric features and spatial information. In mouse cortex, morpho-electric features of GABAergic interneurons covary with the transcriptomic types, and classification based on morpho-electric features is largely consistent with transcriptomic types (Gouwens et al., 2020). In the previous study, only 37 Oxt-positive neurons were identified for transcriptomic classification, and the small sample size may limit the subtypes screening (Romanov et al., 2017). It will advance our understanding about OXTergic system when we conduct single-cell patch-seq (Lipovsek et al., 2021), similar as the research in cerebral cortex (Cadwell et al., 2016; Gouwens et al., 2020).

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